

UNCLASSIFIED

AD NUMBER
ADB270818
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Oct 2000. Other requests shall be referred to US Army Medical Research and Materiel Comd., 504 Scott St., Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 1 Apr 2003

THIS PAGE IS UNCLASSIFIED

AD _____

Award Number: DAMD17-97-1-7346

TITLE: Genotype Phenotype Relationships in Neurofibromatosis 2

PRINCIPAL INVESTIGATOR: Mia M. Maccollin, M.D.

CONTRACTING ORGANIZATION: The General Hospital Corporation
Boston, Massachusetts 02114

REPORT DATE: October 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S.
Government agencies only (proprietary information, Oct 00).
Other requests for this document shall be referred to U.S.
Army Medical Research and Materiel Command, 504 Scott Street,
Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20010925 234

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-97-1-7346

Organization: The General Hospital Corporation

Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Almasha Clea Mum
28/4/97

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 2000		3. REPORT TYPE AND DATES COVERED Annual (30 Sep 99 - 29 Sep 00)	
4. TITLE AND SUBTITLE Genotype Phenotype Relationships in Neurofibromatosis 2				5. FUNDING NUMBERS DAMD17-97-1-7346	
6. AUTHOR(S) Mia M. Maccollin, M.D.					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The General Hospital Corporation Boston, Massachusetts 02114 E-MAIL: maccolli@helix.mgh.harvard.edu				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION / AVAILABILITY STATEMENT DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Oct 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command. 504 Scott Street, Fort Detrick, Maryland 21702-5012.					12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Neurofibromatosis 2 is a genetic disorder characterized by the development of benign nervous system tumors which shows great phenotypic homogeneity within families. The hypothesis of this study is that there is a correlation between the highly variable phenotype of NF 2 and the causative genotype. Progress over the last 3 years has included: 1) development of a database, and clinical consortium; 2) definition of pediatric and spinal cord phenotypic subtypes; 3) validation of exon scanning methods and development of alternative mutation detection methods; 4) documentation of the effect of parental origin on severity; 5) exploration of allelic imbalance as a rapid diagnostic tool.					
14. SUBJECT TERMS Neurofibromatosis				15. NUMBER OF PAGES 29	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

TABLE OF CONTENTS

Introduction-----	4
Body -----	5-18
I. Patient ascertainment and database analysis-----	5-6
II. Molecular analysis -----	6-11
III. Allelic expression of the <i>NF2</i> gene—development of a diagnostic tool-----	11-13
IV. Tables 1 through 7-----	14-18
Key research accomplishments-----	19
Reportable outcomes-----	20-21
Conclusions -----	22
References-----	23
Appendices--	
Screen shots of the NF2 database	

INTRODUCTION

Neurofibromatosis 2 (NF2) is a genetic disorder characterized by the development of bilateral vestibular schwannoma and other nervous system tumors. Both NF2 and neurofibromatosis 1 (NF1) show great clinical variability between individuals with regards to tumor burden, severity of disease and age of onset and death. Despite this overall heterogeneity and unlike NF1, NF2 shows remarkable homogeneity within families, suggesting an effect of the underlying mutation on the resultant phenotype. The hypothesis of this study is that there is a correlation between the highly variable phenotype of neurofibromatosis 2 and the causative genotype. We are investigating this hypothesis under three broad areas:

1. The vast majority of NF2 affected individuals harbor point mutations and small frameshifts of the *NF2* gene. What effect does the type and location of mutation have upon the resulting phenotype?
2. Up to one third of the mutations in individuals with NF2 cannot be detected with exon scanning, and these individuals predominantly carry a mild phenotype. What is the pathogenetic mechanism for this phenotype and what implications does it have for the function of the *NF2* protein product as a tumor suppressor?
3. What is the role of the *NF2* gene in atypical and outlying phenotypes of NF2?

The steps we are taking to investigate this hypothesis are:

1. Identification of a large cohort of affected probands and family members from three major collection sites, clinical characterization of the affected individuals and sample collection of affected and unaffected tissues.
2. Molecular genetic analysis of the *NF2* gene in germline specimens using exon scanning and directed sequencing.
3. Development of alternative strategies of mutational detection for germline specimens not found to have abnormalities by exon scanning.
4. Molecular genetic analysis of the *NF2* locus in somatic (tumor) specimens.

These studies may be expected to shed light on the molecular basis for tumor suppression by the *NF2* gene by identification of critical regions of the transcript and alternative mechanisms of inactivation of this protein. They will also make the molecular diagnostics possible for increasing numbers of both probands and at risk individuals and will clarify the diagnosis of variant phenotypes. Finally, the elucidation of genotype phenotype relationships will aid in the prognostication and management of this devastating disorder.

BODY

I. Patient ascertainment and database analysis:

A. Database utilization and revisions.

The NF2 database is a tool used to facilitate on and off site chart review by non clinicians. During the period of funding, the database was implemented at three major NF2 centers including MGH, The House Ear Institute in Los Angeles (HEI) and the Klinikum Nord Institute in Hamburg, Germany. In the first year of funding, a major database revision was instituted after concerns raised by the peer review panel led us to add a statistician to the project. In the second year of funding, the database was accepted as a part of the separately funded USARMC project on the natural history of vestibular schwannoma in NF2. A second major revision was then completed to allow the database to meet the additional goals of that project. Additional centers have entered data as part of the later project through one of the three major centers. Currently we estimate that our clinical coordinators require one to two hours per patient for initial database entry and 0.5 to one hour for annual database update. Screen shots of the current database, which exists in FileMaker Pro format for both Mac and PC platforms, are placed in the appendix.

B. Databasing and collection of samples

Databasing and collection of samples was significantly impacted by the first revision of the database, since computer entry could not begin until this was completed. As of May, 2000 a total of 301 subjects have been partially or totally databased at the three major centers. A "snapshot" of data regarding three phenotypic features currently under intensive focus are presented in table 1. Collection of samples has been very efficient within the three major collaborating centers. For example, from the period 5/1/99 to 4/30/00 we received and cataloged a total of 159 samples for 116 patients with NF2 and related diagnoses. 28 samples were collected on site at MGH and 43 samples came from the House Ear Institute in Los Angeles. A breakdown of sample type is presented in table 2.

As noted below, a special focus of the last year of funding of this grant was the molecular genetics of classically affected patients who have mutations not detectable by exon scanning methodology. During the granting period we have developed a cohort of 40 such individuals in whom exon scanning did not detect mutation. This cohort includes 30 of the 47 individuals studied directly in the PI's laboratory in whom mutations could not be detected and 10 individuals studied by collaborators to this grant, including the DNA Diagnostics Laboratory. 27 of the 40 individuals are founders, with no preceding history of NF2 in a parent (67.5%). In 2 of these 27

founders, we have collected a sample from an affected offspring to minimize the possibility of mosaicism confounding analysis. In 25 no offspring known to be affected was available for study. Tumor specimens have been collected from a majority of these individuals (or family members) and are currently under study.

C. Phenotypes of severely affected patients

Because NF2 is classically considered a disease of adults, we examined the clinical characteristics of the children entered in the study. A total of 31 children meeting the NIH criteria for NF2 prior to age 17 years were ascertained. 24 had been diagnosed clinically and 7 were diagnosed using molecular methods. Only 3 of 31 patients (10%) presented with hearing loss. 13 children (42%) presented with skin tumors or ophthalmologic abnormalities, the significance of which was often realized because of a positive family history. Vestibular tumors had been detected in 31 of 33 children at ages 1 through 16 and in 6 the tumors were greater than 2 cm at first evaluation. Other intracranial or spinal tumors had been detected in 28 children. The clinical course of these children was highly variable, dependent on both tumor burden at the time of diagnosis and surgical outcome when tumor resection was attempted. Molecular analysis of these patients showed the overwhelming majority to have truncating mutation, as would be expected from our previous studies. An abstract describing these findings was presented at the 27th annual meeting of the Child Neurology Society and a more detailed analysis of these results is presented in MacCollin and Mautner, 1998.

II. Molecular analysis

A. Exon scanning

1. Technical issues

Exon scanning for germline mutation has now been completed on a total of 116 patients directly in the PI's laboratory, with definition of causative mutation in 69 (60%) (table 3). Although this detection rate is comparable to that of other published exon scanning approaches in this transcript, over the past year we have taken two approaches to determine if any of the subjects "unfound" by this technique had small point mutations or frameshifts which were undetected for technical reasons.

A subset of 19 individuals unfound by SSCP analysis in the PI's laboratory were studied by automated sequencing of the exons of the *NF2* gene. The study subjects were primarily those described as unfound in our earliest studies (MacCollin et al., 1994 and Parry et al., 1996) to reduce the potential impact of improvements in SSCP technique and the use of tumor specimens to define mosaicism both of which might improve the detection rate in the currently funded study. Each exon was

amplified from genomic DNA using intronic primers situated at least 20 basepairs from the intron/exon borders. The exons were sequenced in both the sense and antisense direction using dye primer sequencing and an ABI prism 377 sequencer. Each exon run included a known positive control with a heterozygous mutation documented by our previous work. When possible, splice site mutations were used as positive controls, since mutations near the sequencing primers were found to be the most difficult to detect. Sequencing analysis software, (EditView v1.0.1, PE Biosystems), was used to perform the initial basecalling of the sequencing traces. To improve detection of heterozygous changes, all traces were hardcopied in color and manually reviewed by two readers.

Using this protocol, all 16 control mutations in coding exons were detected in both directions by manual review; 20 of 32 control runs were correctly basecalled by the sequencing software. During optimization of primers, a 15/16t polymorphism was detected in intron 6, 42 basepairs 3' to exon 6 in both patient and control samples. A single intronic change of unclear significance (676-4 a to g) and two alterations in codon wobble positions (G720A, C762A) were identified in exon 8. No other mutations or polymorphisms were detected in any patient sample, validating our exon scanning results.

Because of recent reports that indicate DHPLC may be a more sensitive exon scanning approach than SSCP we also sought to directly compare the two in detecting known *NF2* mutations. DHPLC protocols were developed in conjunction with Michael Hepburn, Applications Scientist at Transgenomic, Inc. utilizing a WAVE DNA fragment analysis. Amplicons were prepared to Transgenomic's specifications, which include Betaine monohydrate additive and Platinum *Taq* polymerase. All exons were scanned at least five temperatures and each run included several negative control samples. Chromatograms were read in a blinded fashion by Dr. Hepburn, and mutations were considered present if heteroduplex peaks were seen at any temperature.

Despite multiple attempts, we were unable to amplify paraffin block extracted genomic DNA specimens adequately for analysis, limiting our study to DNA extracted from blood and frozen tumor specimens. No control sample gave a false positive result. Four of the eight mutations were detected, including known heterozygous, hemizygous and mosaic samples. Four low level mosaics were not detected, including a sample which had been detected by SSCP (table 4).

These two lines of investigation suggest that our current SSCP protocols detect a majority of point and small frameshift mutations in this transcript. We have thus focused on alternative mechanisms of mutational detection in our "unfound" patients, described in section B below.

2. Mutations detected by exon scanning; impact on spinal cord tumors

A collaborative study of spinal cord tumors in a subset of the study subjects was undertaken with the NCI (NIH). Complete spinal imaging of 49 patients from 26 families was used to document the extent and type of spinal cord tumors present. 63% of patients were found to have spinal cord tumors which were multiple in 45% of patients. Although intramedullary tumors were fairly common (53% of patients), they became symptomatic only rarely (4 of 49 patients, 8%). Conversely, meningiomas were present in only 18% of patients but accounted for a disproportionate amount of spinal cord compression and surgical procedures.

Potential effects of the underlying genotype were then examined with regards to these findings. Mutations could be identified in 37 patients from 19 families, including nonsense and frameshift (13 patients), splice site alteration (15 patients), and non truncating change (9 patients). Patients bearing nonsense and frameshift mutation had younger ages of onset and diagnosis and greater morbidity from spinal tumors than patients bearing splice site mutations, non truncating mutations or unfound mutations. Particularly intriguing was the observation that no patient with a non truncating or unfound mutation was found to have a spinal meningioma, the tumor type that behaved most aggressively in this study. Effects on ependymomas and nerve sheath tumors were less profound, but still reached statistical significance with regards to both presence and numbers of tumors. A more detailed account of this work is current in press in the journal *Radiology*.

This study illustrates the potential for clinical relevance of our work. For example, these results indicate that patients known to have nonsense and frameshifting mutations should undergo earlier and more frequent spinal cord surveillance, even in the absence of neurological symptoms. Should experimental therapeutics become available, nonsense and frameshift bearing patients with spinal cord tumors would be most likely to benefit from such treatment because they are the most likely to eventually require surgical intervention. Conversely, those patients with unfound and non truncating mutation may be counseled that they are at a decreased risk for the development of symptomatic spinal cord tumors. This later group is less likely to benefit from drug therapy trials, especially those with potential toxicity.

B. Alternative mechanisms of mutational analysis

1. cDNA analysis

A rapid cDNA based assay was developed as an alternative mechanism of mutational analysis for mildly affected patients. Using a cohort of 105 affected unrelated individuals who have undergone mutational analysis using standard

techniques thorough the consortium, a group of control and experimental subjects was constructed. The control group consisted of 14 of the 18 individuals with known splice site mutations. The experimental group consisted of 23 of the 44 predominantly mildly affected patients whose mutations were not detected using exon scanning. RNA was extracted from lymphoblastoid cell lines and cDNA was synthesized by reverse transcription. Amplification of the entire *NF2* coding region was carried out in six overlapping segments.

No control splice site samples produced unexpected size variants and no uniform change was evident in the samples, confirming the lack of alternative splicing in this tissue which has confounded the analysis of tumor tissue. Expected size alterations were seen in 12 of the 14 splice site controls; expression of the splice mutation could not be seen in 2 specimens, consistent with the studies described below showing under expression or lack of expression of mutated *NF2* alleles.

Of the 23 unfound samples tested, 3 (13%) produced an alteration in one or more of the 6 cDNA segments. In 2 of the 3 samples, the altered allele amplified at a much less robust level than did the wild-type allele. All aberrant bands were isolated, re-amplified and sequenced; corresponding genomic DNA changes were then sought. Changes in this group included deletion of exons 2 through 8 (GUS16218), duplication of exons 3 and 4 (GUS16983) and insertion of intronic material between exons 13 and 14 (GUS8873), perhaps due to activation of a cryptic splice site. A more detailed account of these results has been presented in abstract form (Hill et al., Am J Hum Genet 63:A232).

2. Obligate hemizyosity

To search for deletion candidates, we looked for obligate hemizyosity within the cohort of patients unfound by exon scanning. 37 of these 40 individuals have been examined at the highly polymorphic locus D22S929 which lies in intron 1 of the *NF2* gene. 18 of 37 carried a single allele (51% heterozygosity compared to 75% in an unaffected population). Relatively few had potential for very large deletions however, with 4 of 18 having a single allele at the proximal marker D22S193 and 4 at the distal marker D22S430. One individual was non informative at all three markers. 15 individuals have been examined at *NF2*tet, also within intron 1, with 7 of 15 carrying a single allele. All but 1 patient non informative for *NF2*tet was also non informative for D22S929, suggestive of smaller deletions. Current work is focused on the additional markers recently identified in both coding and non coding regions to narrow the field of candidates for Southern analysis. Efforts are also in progress to identify additional family members of potential deletion patients to define obligate hemizyosity.

3. Tumor based analysis

Perhaps the most successful of our mechanisms of alternative analysis has been the use of tumors to define mosaicism in persons with typical (BVS) NF2. As part of this aim, 26 founders were identified in whom no germline *NF2* change could be detected by exon scanning and one or more tumor specimen was available. 25 of 26 had completely asymptomatic parents; the father of a single patient (GUS28350) had multiple pathologically proven neurofibromas, but no symptoms of vestibular dysfunction at age 63 years.

Abnormal exon patterns were detected in 15 of 26 tumor specimens from these patients. Sequence analysis revealed typical truncating mutations including nonsense (11), frameshift (2) and splice site (1) alterations (table 5). A single patient carried a previously undescribed missense mutation in exon 15 of unclear significance.

Loss of heterozygosity of flanking and intra genic markers was seen in 19 of the 26 tumors when compared to blood specimens. When multiple markers were tested across the *NF2* region, all but one tumor showed loss at all informative markers or retention at all informative markers. LOH was seen in 11 of 15 tumors with *NF2* mutation (73.3%) and 8 of 11 tumors without mutation (72.7%). LOH in patient 28350 showed a maternal origin of new mutation, confirming that his father's multiple isolated neurofibromas were unrelated to his *NF2*.

Second tumors were available from 8 of the 15 patients in whom a mutation was detected in the first tumor. In 6 patients (5 of whom had LOH) mosaicism was confirmed with identical changes in the subsequent tumor. In 2 patients (both with no LOH) mosaicism could not be confirmed. Although no SSCP changes had been seen in any blood specimen, sequence analysis, and /or restriction digest analysis were slightly more sensitive in detecting low levels of mosaicism in the blood specimens. These results confirmed mosaicism in 6 of 26 patients whose mutations could not be detected by exon scanning, and were highly suggestive of mosaicism in an additional 6 patients with mutation and LOH (table 6). This group of 12 patients represented 46% of the patients studied. 10 of 12 mutations associated with definite or probable mosaicism involved C to T transitions at CpG islands.

A more detailed phenotypic and molecular examination of mosaicism was possible in the two patients carry C586T because of our previous study of 5 unrelated patients carrying this mutation at a non mosaic level (MacCollin et al., 1996). Although the mosaic patients had a delayed onset of symptoms, their eventual tumor load was similar to that of the non mosaic patients and greater then the average seen in large clinical surveys. T vector cloning of blood samples from these two patients was completed to estimate the level of peripheral lymphocytic mosaicism. A non founder control carrying an identical mutation was used as a control. 12 of 22 clones

tested in the control carried the C586T alteration (54%). In both patients less than 10% of clones (2 of 24 in patient GUS24405 and 2 of 22 in patient GUS28165) carried this mutation.

These results demonstrate that mosaicism accounts for a significant percentage of founders with typical NF2, and mosaic individuals may not manifest changes in blood samples on exon scanning. Molecular genetic analysis of the *NF2* gene should begin in tumor tissue whenever possible, especially when examining a founder. These results were recently presented at the annual meeting of the American Society of Human Genetics (Kluwe et al., Am J Hum Genet 67:2254).

4. Effect of parental origin on phenotype

Two previous studies of NF2 have reported a parent of origin effect on severity in NF2 with the mean age in paternally inherited cases being 24 years and that of maternally inherited cases 18 years. These clinical studies were able only to examine the effect in non-founding generations and may thus have contained biases against more severely affected patients who often do not have children. To circumvent this issue, we sought to examine the effect of parent of origin on phenotype amongst the founding generation. Using primarily LOH studies, we determined the parental origin of mutation in 45 sporadically affected patients with NF2, including six somatic mosaics. New mutation was of paternal origin in 69% of the 45 patients as a whole, 74% of the non mosaics and 33% of the mosaics. A statistically significant effect on severity was seen, with the mean age in paternally inherited cases being 11.3 years and that of maternally inherited cases being 18.2 years. This effect persisted even when corrected for the mitigating effects of known mosaicism. These and other observations regarding parental origin in NF2 are currently in press in the journal *Neurogenetics*.

III. Allelic expression of the *NF2* gene—development of a diagnostic tool

As part of our development of a cDNA based mutation screen (section IIB1), we sought to determine the extent to which mutation bearing alleles are underexpressed in messenger RNA. These studies were initially made possible by the discovery of a relatively frequent polymorphism in the 3' untranslated region (UTR) of the *NF2* gene. This polymorphism consists of a t to c transition which creates a *MaeIII* cleavage site 349 BP downstream from the native stop (t2187c). 200 unrelated individuals (90 with NF2, 24 controls with schwannomatosis, and 86 unaffected controls) were screened for this change and 69 were heterozygous (34.5%).

21 heterozygous specimens from control individuals without NF2 were screened for unequal expression of alleles in cDNA. None were found to significantly differ

from genomic levels of amplification. 29 heterozygous NF2 affected individuals were screened for unequal expression of alleles in cDNA and 24 of 29 showed unequal expression (83%, $p \leq 0.0001$). Differences in allelic expression ranged from 1.8 fold to 20 fold. In 17 patients with unequal expression, the disease bearing allele could be phased with respect to the *MaeIII* polymorphism by family studies, loss of heterozygosity in one or more tumors or analysis of cloned *NF2* cDNAs. The underexpressed allele was the disease bearing allele in all but one specimen. These results, in an expanded form, are presented in Jacoby et al., 1999.

Discovery of the t2187c transcribed polymorphism in the 3'UTR of the *NF2* gene did indeed confirm our hypothesis that there is frequently unequal expression of mRNA from mutant alleles, although it was not usually to an extent that would preclude cDNA screening. More importantly, however, it brought to our attention the possibility that unequal expression might be of diagnostic use as this assay was far more simple and rapid than the mutational detection protocols currently used for diagnosis and appeared to have great specificity and acceptable sensitivity with regards to affected and unaffected status. To further explore the use of unequal expression as a diagnostic assay, primers were created to amplify 3 of the 4 biallelic polymorphisms in the 5' UTR described by Zucman-Rossi et al. (1998). Each of these polymorphisms creates or destroys a restriction enzyme recognition site in the less frequent allele. The fourth polymorphism in the 5' UTR (ATG-643, ins 1 BP (T)) cannot be directly assayed by restriction cleavage. Specific primers that amplify either the 6T or 7T variant, linked with an *EcoRI* or *HindIII* linker to a 20 BP tail are used in equalmolar amounts with a 3' primer 108 BP 3' to the polymorphism site (see appended figure). Digestion with *EcoRI* or *HindIII* produces separation of the alleles best visualized on acrylamide gels using radiolabeling or the Pharmacia GenePhor separation system. Preliminary application of these assays to a cohort of 20 individuals revealed heterozygosity ranging from 25 to 40 %.

Over the past year, we have explored the less complex option of quantitative assessment of these assays using area-under-curve values generated by an automated sequencer. A single primer for each assay was re synthesized with a 5' end 6-FAM (blue) label. Control samples consisting of genomic DNA known to be homozygous at the 8881 polymorphism were amplified with the labeled primer, and digested with *FseI*. After visual inspection on agarose gels to insure complete digestion and estimate the amount of product, samples with fixed ratios of upper to lower alleles were analyzed. The area under curve ratios were qualitatively appropriate, but underestimated the over represented allele. Similar results were obtained with mixing experiments using homozygous samples at the 8787 polymorphism. We then examined two cell lines known to have unequal expression (GUS7865 and 16209).

The ratio of alleles in cDNA was qualitatively changed from that of genomic amplification, but again suggested underestimation compared to previous experiments (table n). Fresh blood samples from an unaffected (DAV224) and affected (GUS29232) individual showed similar results. As we develop additional assays in the UTR and apply these to larger numbers of patient and control samples, the follow areas will be addressed:

- determination of the sensitivity and specificity of qualitative versus quantitative analysis and area under curve versus audioradiography.
- test/retest assessment at the level of sampling, extraction, reverse transcription and amplification
- effect of RNA source (lymphoblastic cell line versus lymphocytes and effect of time delay in extraction as might be incurred in transport)

	N	hearing loss			spinal cord tumor		ocular complication		
		percent	average age of onset (years)	range age of onset (years)	radiographic	required surgery	cataract	retinal change	dysconj. gaze
MGH	68	69%	24.4	5-51	57%	27%	30%	10%	20%
HEI	84	44%	25.3	3-76	44% ¹	6%	NA	NA	NA
Hamburg	149	67%	23.7	0-63	89%	32%	68%	17%	14%
Evans, 92	120	98% ¹	24	4-50	26%	NG	38%	NG	10% ³
Parry, 94	63	87%	25.7 ²	12-70 ²	67% ¹	14%	81%	8%	NG
Mautner, 96	48	NA	NA	NA	90%	26% ³	63%	13%	5%

Table 1. Clinical features in NF2 patients currently in database, versus large published clinical surveys. Radiographic appearance of spinal cord tumor is the percent of patients with one or more spinal cord tumors by MRI of any spinal segment, but except for the Hamburg group, not all spinal cord segments had been imaged in all reported or studied patients. NA--not available (for the past 7 years, HEI patients have not had routine ophthalmologic evaluation). 1--of a subset of individuals in the group. 2--includes all symptoms of VS, not just hearing loss. 3--separate study by the same authors.

Fresh blood specimen—75
Frozen tumor specimen—15
Fresh tumor specimen—9
Skin biopsy for culture—9
Pathology block—21
Touch preparation made from fresh tumor—10
Cord blood sample—1
Pre extracted genomic DNA—4

Table 2. Sample collection over a one year period of funding (5/1/99 to 4/30/00). Some study participants donated more than one sample (for example, both tumor and blood) during this time period.

Mutation type	N	%
Nonsense	30	26
Frameshift	15	13
Splice site	18	16
Non truncating	6	5
Unfound	47	40
TOTAL	129	100

Table 3. Results of exon scanning in the PI's laboratory.

Sample ID	Mutation	Level	SSCP	DHPLC
99-693	168 C to T	mosaic	detected	not detected
24405	586 C to T	mosaic*	not detected	not detected
28165	586 C to T	mosaic*	not detected	not detected
97-494	586 C to T	heterozygous	detected	detected
DAV179	810+2 t to c	hemizygous tumor	detected	detected
28001	810+2 t to c	mosaic	detected	detected
28349	1396 C to T	mosaic	not detected	not detected
DAV139	1396 C to T	heterozygous	detected	detected

Table 4. Comparison of mutational detection by our SSCP protocol to DHPLC utilizing a WAVE DNA fragment analyzer. *Low level mosaics as defined by tumor block analysis and T vector cloning.

ID	Exon	Sequence change	Predicted effect
99-707	15	A1640 A to G	Glu547 Gly
13772	9	del 1 bp (G)	frameshift
17907	11	<i>1021 C to T</i>	Arg341 X
24405	6	<i>586 C to T</i>	Arg196X
25974	4	<i>439 C to T</i>	Gln147X
27587	13	<i>1396 C to T</i>	Arg 466X
28165	6	<i>586 C to T</i>	Arg196X
28349	13	<i>1396 C to T</i>	Arg466X
28350	2	<i>169 C to T</i>	Arg57X
28964	12	1340 g to a	splice donor
28967	3	260/1 to 284/5 del 25 BP	frameshift
K13	6	<i>586 C to T</i>	Arg 196X
K358	2	<i>193 C to T</i>	Gln65X
K72	2	<i>169 C to T</i>	Arg 57X
K74	6	<i>586 C to T</i>	Arg 196X

Table 5. Sequence changes detected in first tumors. Alterations at "warm spots" of mutations previously reported in multiple unrelated individuals are printed in *italics*.

ID	First tumor		Second tumor		Conclusion
	Sequence	LOH	Sequence	LOH	
99-707	missense (?)	yes	NA	NA	presumed
13772	frameshift	retained	NA	NA	inconclusive
17907	nonsense	lost*	nonsense	lost*	definate
24405	nonsense	retained (?)	586 C to T	retained	definate
25974	nonsense	lost	NA	NA	presumed
27587	nonsense	retained	NA	NA	inconclusive
28165	nonsense	lost	nonsense	lost	definate
28349	nonsense	lost	nonsense	lost	definate
28350	nonsense	lost	NA	NA	presumed
28964	splice	retained	mutation not detected	lost	inconclusive
28967	frameshift	lost	NA	NA	presumed
K13**	nonsense	lost	nonsense	NA	definate
K358	nonsense	lost	NA	NA	presumed
K72	nonsense	lost	NA	NA	presumed
K74	nonsense	lost	nonsense	NA	definate

Table 6. Results of molecular analysis of second tumors. Definate mosaics had two tumors which shared the same mutation. Presumed mosaics had no second tumor available, but had a single tumor with mutation and LOH. *LOH by intragenic deletion. **Previously reported in Kluwe et al., 1998.

ID	status	assay	ratio	t2187c ratio
GUS7865 (cell line)	affected	8881	1.99	14.6
GUS16209 (cell line)	affected	8881	2.13	12.1
DAV224 (fresh blood)	unaffected	8787	1.11	NA
GUS29232 (fresh blood)	affected	8787	1.99	NA
GUS29232 (fresh blood, retest)	affected	8787	1.99	NA

Table 7 Application of 5'UTR assays to patient samples using area-under-curve assessment. Ratios are normalized to paired genomic DNA samples. t2187c ratios are from Jacoby et al., 1999 and derived from phosphorimaging analysis.

KEY RESEARCH ACCOMPLISHMENTS

- Development of an NF2 database and consortium of specialty centers
- Definition of the pediatric phenotype of NF2
- Validation of current exon scanning methods
- Development of alternatives to exon scanning, especially for mosaic individuals
- Definition of the effect of genotype on the morbidity of spinal cord tumors
- Discovery of the effect of parental origin of new mutation on the severity of disease in sporadically affected NF2 patients.
- Development of allelic expression assays

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

MacCollin M, Mautner VF. The diagnosis and management of neurofibromatosis 2 in childhood. *Seminars in Pediatric Neurology* 5:243-252 (1998)

Hill E, Jacoby LB, Rogers A, MacCollin M. Mutational analysis of neurofibromatosis 2: alternatives to exon scanning. *Am J Hum Genet* 63:1330 (1998)

Jacoby LB, MacCollin M, Parry D, Kluwe L, Lynch J, Jones D, Gusella J. Allelic expression of the *NF2* gene in neurofibromatosis 2 and schwannomatosis. *Neurogenetics* 2:101-108 (1999)

Andrade A under the mentorship of Mia MacCollin. Mosaicism in the *NF2* tumor suppressor gene. First place finish in Boston City Wide Science Fair, April 10-11, 1999 (also presented at the New England Regional Science Symposium, April 11, 1999 and the Massachusetts State Science Fair, April 30-May 1, 1999)

Friedman JM, Gutmann DH, MacCollin M, Riccardi V. *Neurofibromatosis: Phenotype, Natural History, and Pathogenesis*, 3rd Ed. The Johns Hopkins University Press, 1999.

Kluwe L, Mautner V, Xin W, Jacoby LB, Willett CJ, MacCollin M. Molecular determination of somatic mosaicism in typical *NF2* patients. *Am J Hum Genet* 67:2254 (2000).

Kluwe L, Mautner V, Parry D, Jacoby L, Baser M, Gusella J, Davis K, Stavrou D, MacCollin M. The parental origin of new mutations in neurofibromatosis 2. *Neurogenetics*, in press.

Patronas N, Courcoutsakis N,, Bromley C, Katzman G, MacCollin M, Parry D. Intramedullary and spinal canal tumors in patients with Neurofibromatosis 2: MR Imaging findings and correlations with genotype. *Radiology*, in press

Cell lines, tissue or serum repositories:

From the period 5/1/99 to 4/30/00 we received and cataloged a total of 159 samples for 116 patients with *NF2* and related diagnoses. A breakdown of sample type is presented in table 2.

Informatics:

NF2 germline mutation map, initially compiled June, 1995, most recent revision August, 1999, accessed on the World Wide Web at: <http://neuro-trials1.mgh.harvard.edu/nf2/> References for the map, including unpublished sources of information, are listed at the map site.

Funding applied for based on work supported by this award:

Title: Genotype Phenotype Relationships in Neurofibromatosis 2

PI: MacCollin, Time commitment: 33%

Supporting agency: National Institutes of Health

Duration: 2/1/01 to 1/31/06

Level of funding: \$225,000 direct costs in the first year

The goal of this project is to explore the relationship between the highly variable phenotype of neurofibromatosis 2 and the underlying genotype. The proposal is a direct extension of the current project, and expands the scope in several areas.

Decision is pending.

CONCLUSIONS

1. The NF2 database provides comprehensive and reliable access to important NF2 phenotypic features for this and other funded studies. Interaction between this study and the recently funded Army grant on the natural history of vestibular schwannoma should greatly facilitate the power of both studies.
2. Children with NF2 frequently harbor large vestibular tumors, but are more likely to come to medical attention because of skin tumors and ocular abnormalities. Molecular diagnosis of at risk children (offspring of a parent with bilateral vestibular tumors) is an important tool for early recognition, but of equal utility is a careful dermatological and neurological evaluation.
3. Our current exon scanning protocol appears to detect a majority of the small point and frameshifting mutations in this transcript. New technologies should be appropriately assessed as they become available.
4. Alternative mechanisms of analysis, such as cDNA based screening is valuable in revealing some of the mutations not detected by standard, genomic based exon scanning. Mosaicism appears to account for a significant percentage of founders with typical NF2 however. Molecular genetic analysis of the NF2 gene should begin in tumor tissue whenever possible, especially when examining a founder.
5. NF2 patients bearing nonsense and frameshifting mutations have greater morbidity from spinal cord tumors. Consequently, they should undergo earlier and more frequent spinal cord surveillance, even in the absence of neurological symptoms. Should experimental therapeutics become available, nonsense and frameshift bearing patients with spinal cord tumors would be most likely to benefit from such treatment because they are the most likely to eventually require surgical intervention.
6. There is a statistically significant effect of the parental origin of new mutation on the severity of disease in sporadically affected NF2 patients. This effect persists even when correcting for the mitigating effects of known mosaicism.
7. Unequal allelic expression of mutated NF2 loci may be harnessed as a valuable, rapid and inexpensive diagnostic tool. Further work is needed to determine the sensitivity and specificity of this approach.

REFERENCES

- Hill E, Jacoby LB, Rogers A, MacCollin M. Mutational analysis of neurofibromatosis 2: alternatives to exon scanning. *Am J Hum Genet* 63:A232, 1998
- Jacoby LB, MacCollin M, Parry D, Kluwe L, Lynch J, Jones D, Gusella J. Allelic expression of the *NF2* gene in neurofibromatosis 2 and schwannomatosis. *Neurogenetics* 2:101-108 (1999)
- Kluwe L, Mautner V, Xin W, Jacoby LB, Willett CJ, MacCollin M. Molecular determination of somatic mosaicism in typical NF2 patients. *Am J Hum Genet* 67:2254 (2000).
- Kluwe L, Mautner VF. Mosaicism in sporadic neurofibromatosis 2 patients. *Hum Mol Genet.* 7:2051-5 (1998).
- Kluwe L, Bayer S, Baser M, Hazim W, Wolfgang H, Funsterer C, Mautner VF: Identification of *NF2* germ-line mutations and comparison with neurofibromatosis 2 phenotypes. *Hum Genet* 98:534, 1996.
- MacCollin M, Ramesh V, Jacoby L, Louis D, Rubio MP, Pulaski K, Trofatter J et al: Mutational analysis of patients with neurofibromatosis 2. *Am J Hum Genet* 55:314, 1994.
- MacCollin M, Braverman N, Viskochil D, Rutledge M, Davis K, Ojemann R, Gusella J, Parry DM. A point mutation associated with a severe phenotype of neurofibromatosis 2. *Ann Neurol.* 40:440-5 (1996).
- Parry D, MacCollin M, Kaiser-Kupfer M, Pulaski K, Nicholson HS, Bolesta M, Eldridge R, Gusella J: Germline mutations in the neurofibromatosis 2 (*NF2*) gene: Correlations with disease severity and retinal abnormalities. *Am J Hum Genet* 59:529, 1996.
- Zucman-Rossi J, Legoux P, Der Sarkissian H, Cheret G, Sor F, Bernardi A, Cazes L, Giraud S, Ollagnon E, Lenoir G, Thomas G. *NF2* gene in neurofibromatosis type 2 patients. *Hum Mol Genet.* 7:2095-101 (1998).

Personnel receiving pay from the research effort:

T. Smith
M. Badawy
M. MacCollin
A. Masuda
Z. Davenport
S. Acierno
C. Willett
E. Hill
A. Andrade
T. Buckley
E. Ballon
A. Clark
R. Colen
C. Yeoh
B. Heinrich



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 Apr 03

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218


SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession document numbers be changed to "Approved for public release; distribution unlimited." Copies of these reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB277986
ADB263450
ADB267669
ADB277564
ADB261754
ADB257280
ADB283722
ADB249627
ADB282841
ADB266235
ADB283529
ADB283519
ADB256683
ADB262564
ADB271045
ADB283537
ADB257204
ADB283513
ADB281571
ADB262777
ADB270818
ADB283748
ADB274588
ADB283788
ADB259015
ADB266031